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AWARD NUMBER: W81XWH-05-C-0004

TITLE: Targeted Therapies for Myeloma and Metastatic Bone Cancers

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CONTRACTING ORGANIZATION:
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REPORT DATE: June, 2009

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Ft. Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☑ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-2009	Annual	18 Jan 2008 - 17 May 2009

4. TITLE AND SUBTITLE
Targeted Therapies for Myeloma and Metastatic Bone Cancers

5a. CONTRACT NUMBER
W81XWH-05-C-0004

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Southwest Research Institute

San Antonio, TX 78228

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research and
Materiel Command
Ft. Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

- Developed methods to radiolabel polymer nanoparticles; to our knowledge, this is the first time this has been accomplished.
- Quantified functional groups available for ligand conjugation using OPA amino reagent.
- Developed alternative assay to confirm affinity of bone-targeting nanoparticles to hydroxyapatite substrates.
- Demonstrated *in vitro* stability of radiolabeled nanoparticles.
- Successfully transferred our nanoparticle preparation protocols to another facility to support *in vivo* biodistribution studies.
- Started *in vivo* biodistribution studies to validate ability of our radiolabeled nanoparticles to be imaged for up to 48 hours and further confirming our protocol methods to study bone-targeting nanoparticle biodistribution via radio-imaging.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

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I. INTRODUCTION

The goal of this project is to determine in preclinical studies, the potential of skeletally targeted proteasome inhibitors as an efficacious and selective treatment for myeloma. We have found that several proteasome inhibitors are effective against both human and murine myeloma cells in culture. However, as with any proteasome inhibitor, there are serious concerns over the potential systemic effects and toxicity. Our hypothesis is that bone-targeting nanocarriers can preferentially accumulate in the skeleton and locally release proteasome inhibitors to impair the capacity of myeloma cells to survive and grow *in vivo,* thereby reducing the formation and growth of tumor-induced lytic bone lesions. Proteasome inhibitors are otherwise not selective to bone and their therapeutic-toxic window may be narrow when administered systemically. The scope of this project is to validate our hypothesis. The major tasks are:

1) Formulate and characterize drug-containing, bone-targeting nanocapsules.
2) Determine the *in vivo* biodistribution of bone-targeting nanocapsules.
3) Evaluate the efficacy of bone-targeted delivery of proteasome inhibitors on myeloma tumor progression using the myeloma 5TGM1 murine model.

The outcomes of this research will be significant. The study will demonstrate the preferential biodistribution of nanocarriers specifically designed to target and adhere to bone matrices. It will further show that these same nanocapsules can selectively deliver a specific and potent proteasome inhibitor to skeletal sites to act as an anti-myeloma agent. Targeted bone delivery has several potential benefits, including reduced systemic exposure, increased efficacy in the targeted microenvironment, and the ultimate opportunity for reverse catastrophic disease processes. Furthermore, targeted delivery to bone has several additional significant application opportunities in the areas of osteoporosis, fracture healing, cartilage repair, and tissue engineering.

II. BODY

The project is broken down into the following tasks:

1) Formulate and characterize drug-containing, bone-targeting nanocarriers
2) Determine the *in vivo* biodistribution of bone-targeting nanocarriers
3) Demonstrate the efficacy of bone-targeted delivery of proteasome inhibitors on myeloma tumor progress

Task 1 was scheduled to occur during years 1 and 2.
Task 2 was scheduled to occur during years 2 and 3 with some overlap with Task 1.
Task 3 is scheduled to occur during the last year and half of the project.

1) Task 1 is focused on the development of the bone-targeting nanoparticles and is broken down into the following subtasks:

- Selection of proteasome inhibitors for *in vivo* studies. Complete and reported in the first annual report.
- Formulation and characterization of bone-targeting nanoparticles. Complete and discussed in the first and second annual report.
- Demonstration of adhesion of bone-targeting nanoparticles to bone-like substrates *in vitro*. Preliminarily discussed in the second annual report and discussed further in this annual report.
- Formulation of proteasome inhibitors into bone-targeting nanoparticles. Preliminarily discussed in the second annual report. Additional work was postponed until completion of biodistribution studies discussed in Task 2.

The bulk of the work during this year of the project was focused on further development and characterization of bone-targeting nanoparticles. This consisted of the following tasks:

a) Radiolabeling of nanoparticles
b) Quantifying ligand attachment to nanoparticles
c) *In vitro* binding of ligated nanoparticles to bone-like surfaces
d) Synthesis of targeting polymers
e) Cell based testing of drug loaded nanoparticles

Formulation of Nanoparticles

Nanoparticles were prepared by emulsification method, characterized for particle size as a function of polymer concentration (Figure1), or as a function of PEG concentration (Figure 2). Figure 3 shows a SEM microphotograph to confirm the particle size. We determined zeta potential (surface charge of nanoparticles) as a function of PEG concentration and the results are shown in Figure 4. The zeta potential decreases with increasing concentration of PEG. Particles made with 100% PLGA-PEG have the less negative charge and are almost neutral. Cryoprotectants were required to avoid particle agglomeration during lyophilization. Figure 5 shows the change in nanoparticle size as a function of a cry-protecting agent (F-68) ratio to PLA-PEG.

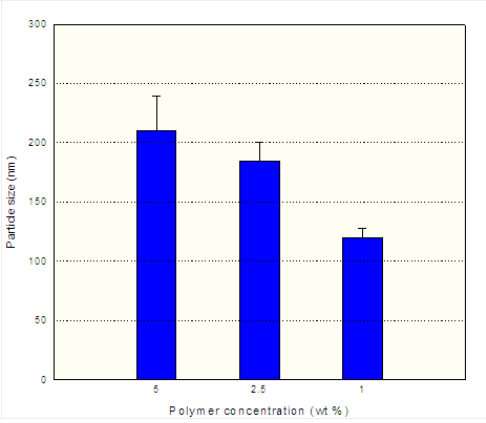

Figure 1. Nanoparticles size as a function of polymer concentration

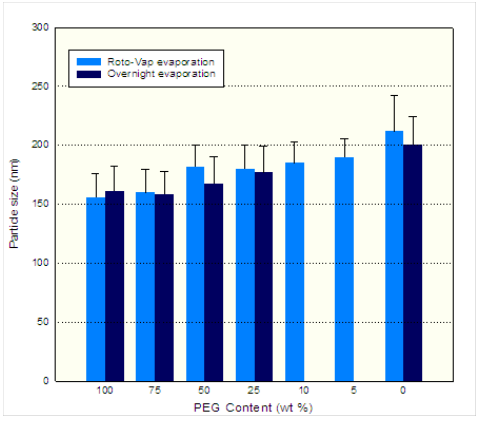

Figure 2. Nanoparticle size as a function of PEG concentration

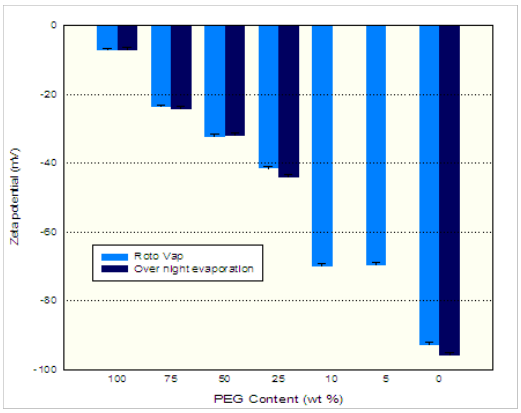

Figure 3. SEM picture (scanning electron micrograph)

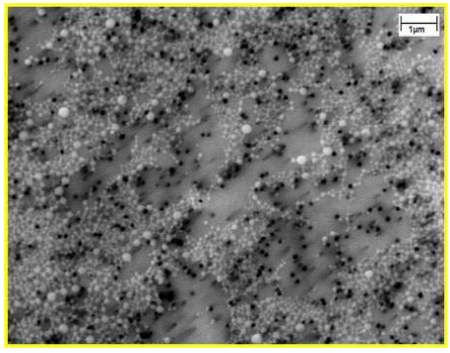

Figure 4. Zeta potential as a function of PEG concentration

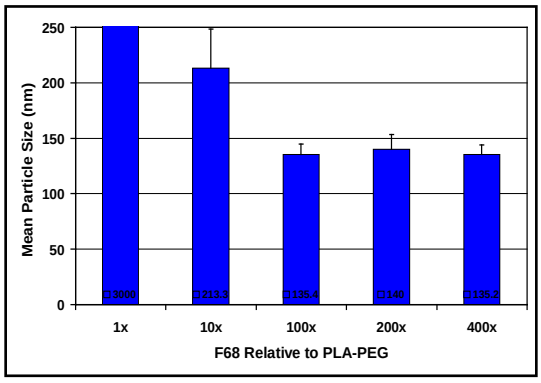

Figure 5. Nanoparticle size after freeze drying as a function of the of F68 to PLA-PEG ratio (cryo- preservation agent)

Radiolabeling of Nanoparticles

During the past year we further improved our radiolabeling protocols using a multiple variable approach that included reducing labeling time, decreasing particle size, increasing PEG content, and reducing surface charge. All of these strategies to optimize radiolabeling will generate better material for the *in vivo* animal studies.

Our initial approach was to develop methods of radiolabeling of nanoparticles (Task 2.1, Proposal Section 5.5.1) in preparation for biodistribution studies planned in Task 2. We selected the gamma emitter ^{99m}Tc, with a 6.5 hr half-life, based on the experience of our collaborators at The University of Texas Health Science Center, San Antonio (UTHSCSA). ^{99m}Tc is hydrophilic and provided as an aqueous solution from the cyclotron source. We originally proposed chelating this radionuclide with a lipophilic chelator, mirroring methods to label liposomes, to facilitate encapsulation using our simple precipitation protocol. However, this approach was not successful. After examining several commercial chelators,

we found both -- the chelation and the encapsulation efficiency to be very dependent on the 'quality' of the radionuclide. ^{99m}Tc is subject to oxidation, which affects chelation efficiency and, in turn, affects encapsulation efficiency. In most cases, encapsulation efficiency was less than 20%, which we deemed insufficient. Subsequently, we explored the conjugation of ^{99m}Tc to reduced proteins, such as bovine serum albumin (BSA), to improve the loading efficiency into nanoparticles (Figure 6). This yielded encapsulation efficiencies of 90% or greater. Payload stability was monitored over a 24-hour period and was found to be nearly 100%. Figure 7 shows *in vitro* stability in serum and in PBS, and Figure 8 demonstrates *in vivo* stability of non-targeted nanoparticles (most of the label is in the liver with a very small amount seen in the bladder; this fraction representing free ^{99m}Tc *in vivo*.

However, we had concerns about residual reducing agent used to activate the protein interfering with coupling of bone-targeting ligands to functionalized nanoparticles.
Therefore, we modified the radiolabeling method to use a commercial reducing gel that can be removed from the protein preparation by simple centrifugation. The newly reduced, clean protein can then be used to complex the radionuclide for encapsulation using the same procedure described previously. The encapsulation efficiency is unaffected by this slightly altered approach. Furthermore, this modified method avoids a lengthy column separation process that diminishes the amount of available radioactivity.

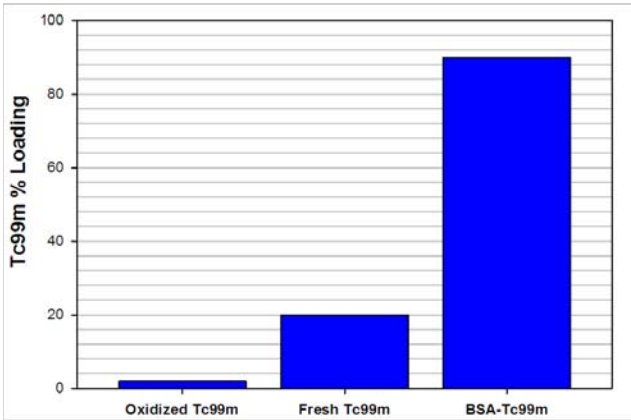

Figure 6. Radio labeling of nanoparticles using oxidized, fresh or conjugated Tc99 into BSA

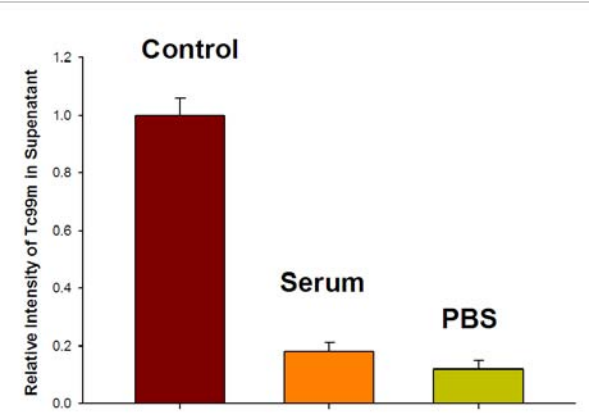

Figure 7. Relative stability of ^{99m}Tc label nanoparticles in vitro after 24 hours incubation

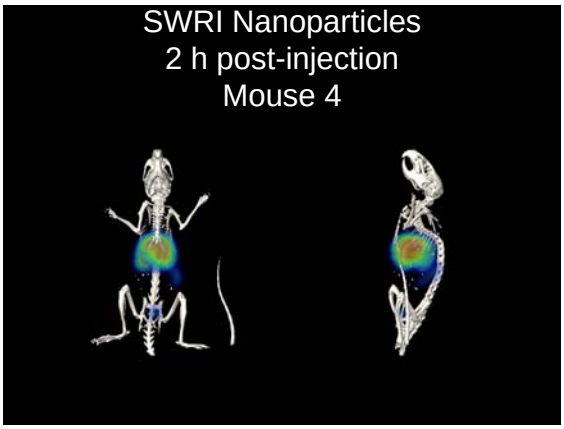

Figure 8. Stability of ^{99m}Tc nanoparticles in vivo measured by SPECT technique after 2hours post-injection

Ligand Quantification

After testing different methods of direct ligand quantification, we finally developed our own procedure to quantify the ligand content directly using acid hydrolysis and colorimetric detection, a method similar to amino acid analysis. On previous attempts we tried unsuccessful NMR techniques to quantify the ligand. In retrospect, the size of the polymer relative to the small size of the ligand, made that approach very difficult.

This year we have been using acid hydrolysis followed by primary amine detection with OPA reagent (O-Phtalaldehyde). In this method, nanoparticles are treated by controlled acid hydrolysis a procedure similar to amino acid analysis of proteins. The released amino ligand is quantified using a sensitive fluorescent amino terminus reagent (OPA). This method was applied on nanoparticles followed by surface ligand conjugation using malamide chemistry or on nanoparticles made with polymer with targeting ligand already conjugated on the starting materials before nanoparticle formation.

Using the above procedure, we have been able to detect the presence of the ligand in all of the targeting polymers synthesized so far. Using this method we found that the conjugation efficiency was a variable that depends on the route of synthesis, and it varies from the low 5% for ASP4 ligand to 25% efficiency for BP ligand. This method is now a standard technique for ligand quantification and we used this technique to guide the ligand conjugation.

Hydroxyapatite Binding (HAp Binding)

As a first approach, we created ligand-nanoparticle conjugates containing functionalized surfaces. After a cleanup procedure, the nanoparticles are tested for HAp binding. To improve the sensitivity of this technique, we used radiolabeled nanoparticles. One added benefit is that being able to demonstrate HAp binding (using the same technique and particle formulation that later will be tested *in vivo*) will reduce the chances of artifacts and non-reproducible results. So far, we have shown preferential adhesion of ligand-containing nanoparticles to hydroxyapatite substrates *in vitro*.

On a second approach, nanoparticles made with polymers already containing ligands (PLGA-PEG-BP and PLGA-PEG-ASP4) blended with different ratios of PLGA-PEG, have been used to evaluate HAp *in vitro* binding. This approach was developed this year thanks to the successful synthesis of the starting materials as discussed below (see synthesis). However, the binding experiments performed using this approach are negative so far. It is possible that the surface ligand exposure using pre-conjugated polymers is much lower than post conjugation in already-made nanoparticles. We still have several polymers that need to be tested before reaching a final decision on using this second approach for *in vivo* testing. Figure 9 and Figure 10 show *in vitro* binding data using post conjugation methods (particles are made with a PLGA-PEG-MAL); the reactivity of malamide (MAL) is used post particle formation to conjugate the bone targeting ligand. This approach seems to be working and is the method used for all the *in vivo* studies carried out up to now, as shown below. Figure 9 shows binding to HA using a bisphosphonate (BP) or poly aspartic acid (ASP6) ^{99m}Tc labeling as a tracer. Figure 10 shows HA binding results for ASP6 ligand using a dye molecule as a tracer. The two methods demonstrate specific binding with some level of background binding (clear bars). The background binding can be due to the entrapment of nanoparticles with HA particles.

It is important to mention that using pre-made polymers with ligand could in theory simplify all the development of this technology. This was contemplated in the initial proposal; however, this approach has been more difficult than it was anticipated.

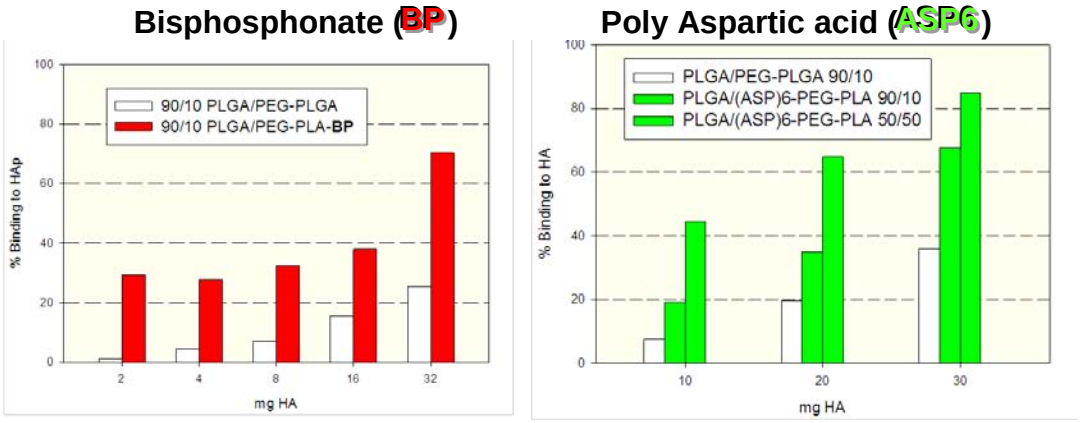

Figure 9. In vitro nanoparticle binding for two different ligands (BP, Bisphosphonate and ASP6 polymeric form of aspartic acid).

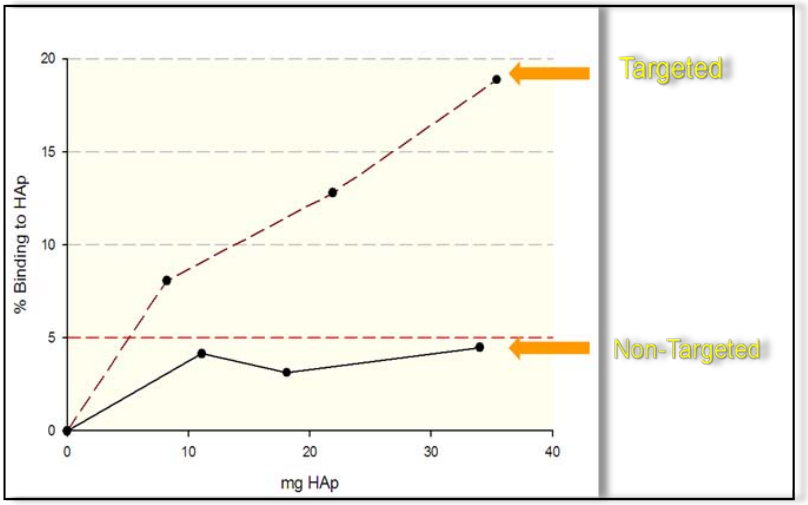

Figure 10. Hydroxyapatite binding using a dye as a tracer, (there is a small (~5%) binding of the non-targeted control)

Synthesis

Our initial work to synthesis of PLGA-PEG-BP and PLGA-PEG-ASP4 had generated polymers with a 5-25% yield as quantified using the OPA technique. To improve the yield, we developed alternative routes that should result in even higher efficiency of the final product.

We feel that we have been able to generate and test multiple polymer chemistries will provide long run benefits, especially when we start with to animals studies. For example, different PEG tether lengths could make a big difference on the ligand exposure, something already described in related literature. Also, the ability to provide ready to use polymers as off-the-shelf reagents with the targeting ligand already conjugated will provide a simplified solution for bone targeting technologies.

Cell Based Testing of Velcade-Loaded Nanoparticles

Recently, the proteasome inhibitor, bortezomib (Velcade), approved to treat multiple myeloma in humans, has become commercially available for research purposes. This compound was loaded on nanoparticles and tested *in vitro* using the 5TMG1 myeloma cell line. This cell line was obtained from our collaboration with Dr. Greg Mundy's lab at Vanderbilt in Nashville, Tennessee. Preliminary results indicate that Velcade remains active after encapsulation (Figure 11). Figure 12 shows free versus encapsulated drug measured by cytotoxicity assay. Also, controlled release is shown in Figure 12 with cumulative drug release reaching 100% at Day 7.

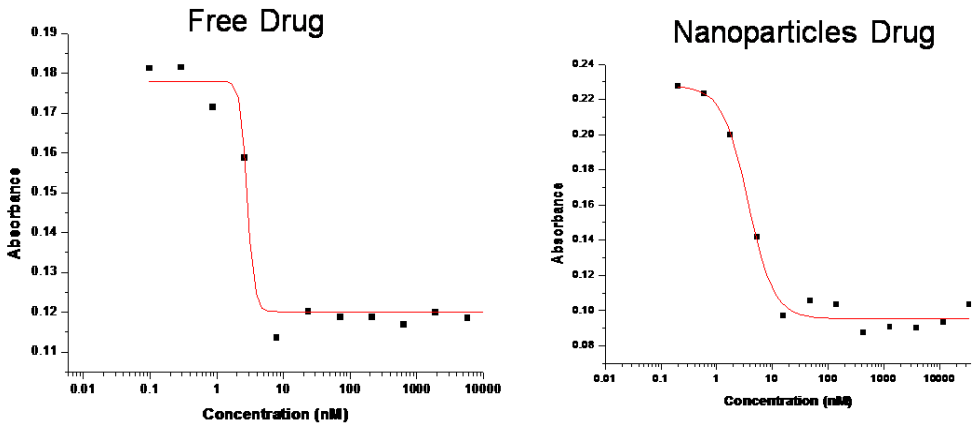

Figure 11. Cytotoxicity of Velcade (Bortezomib) free drug or nanoparticle loaded drug on 5TMG1 Myeloma cells line after 24 hours treatment

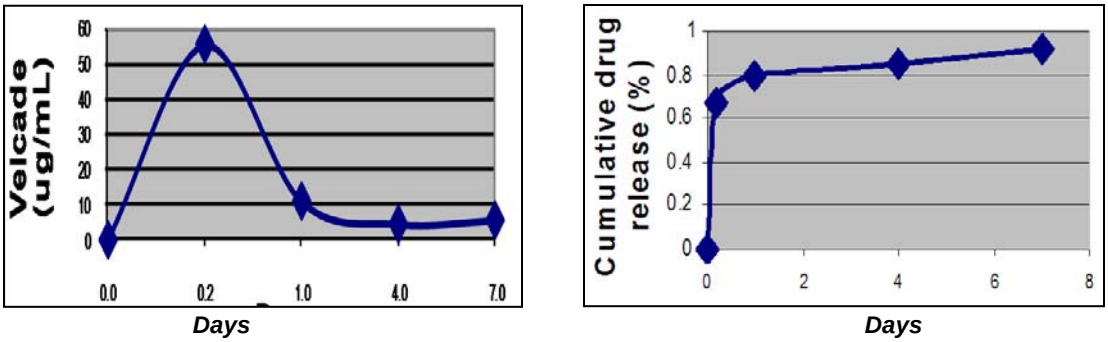

Figure 12. Drug release form nanoparticles (Velcade) in phosphate buffer expressed as µg/ml (left panel) or cumulative percentage of drug released (right panel)

Velcade: Recommended clincal dose = 1.3 mg/m^2,

Rat equivalent = 0.2 mg/kg in rat

Drug loading: 0.5-1.5% (5-15 ug/mg) for tested formula

Nanoparticles usage: typical 2 mL/kg injection at 10 mg/mL concentration can reach the required dosage in animal

2) Task 2 is concerned with determining the *in vivo* biodistribution of bone-targeting nanoparticles in a myeloma mouse model. The task is broken down into the following subtasks:

- Prepare radio-labeled bone-targeting nanoparticles, complete.
- Conduct *in vivo* biodistribution assay.

Task 2 has been delayed by operational and technical difficulties. We conducted initial animal trials in late November/early December 2007, which allowed us to confirm our *in vivo* protocols and test initial nanoparticle formulations. However, this year the task was delayed due to serious illness of our collaborator, Dr. Mundy. Particle formulations and radiolabel contents were more than sufficient to permit *in vivo* imaging, but, the initial particle size of 180 nm proved too large to support long-term distribution. This led to further development to reduce the particle size, which raised some previously unknown issues with nanoparticle formulation by-products on smaller nanoparticle colloidal stability. This forced us to resolve these issues before proceeding with additional *in vivo* studies. We plan to have *in vivo* biodistribution studies completed by late March 2010, and we will start efficacy studies in April 2010.

Initial exploratory biodistribution studies were done using 120 nm and 200 nm nanoparticles in non-diseased mice. Biodistribution studies were performed on 6 animals per group; freshly made nanoparticles loaded with ^{99m}Tc were injected in mice by tail vain injection. Figure 13 shows bone biodistribution. Figure 14 shows biodistribution in several tissues and Figure 15 shows blood levels from 0-44 hours. Bars in Figures 13-15 represent mean ±SD (n=6).

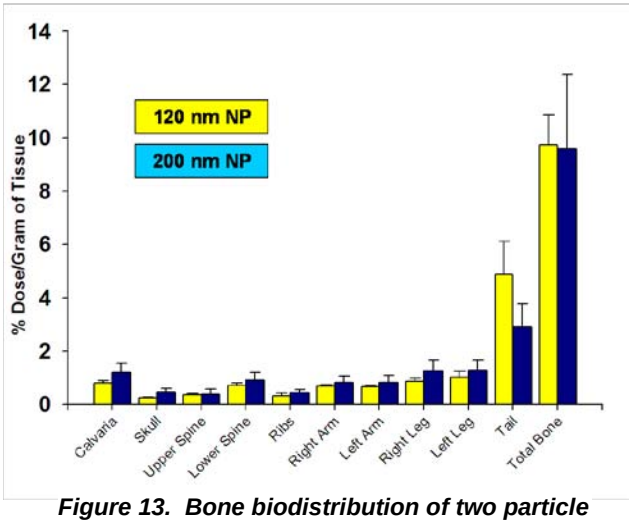

Figure 13. Bone biodistribution of two particle sizes (120 and 200 nm

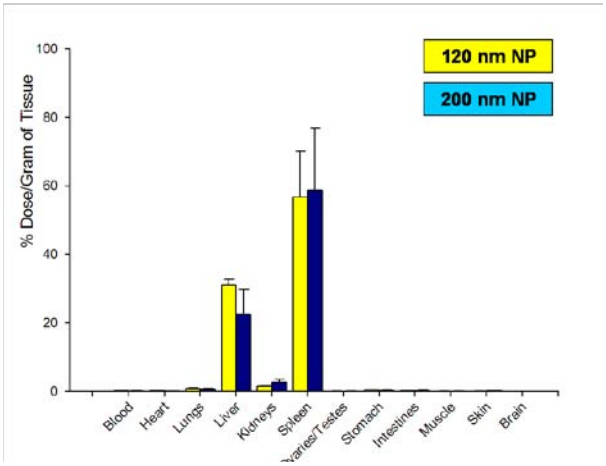

Figure 14. Whole body biodistribution

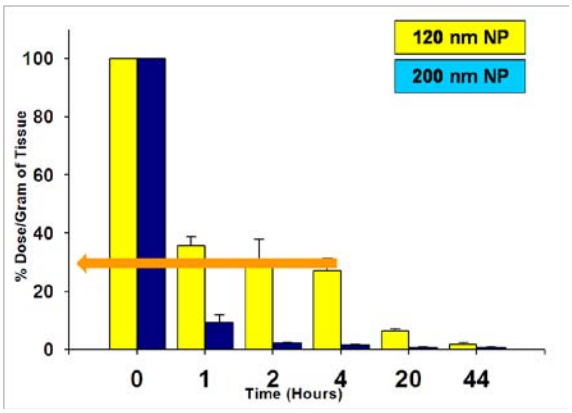

Figure 15. Blood levels for two particle sizes

Because of the reduced blood circulation time on the initial experiments, we decided to modify the nanoparticle formulation method and the results are shown below. Compared with the above results, we observe now a more prolonged half-life for the non-targeted nanoparticles. However, the targeted particles have reduced time in blood. For this reason, we need to explore one last modification to the formulation. This time we will add a different surface modifier (deacetylated chitosan).

The figures below show biodistribution performed on 6 animals per group; freshly made nanoparticles loaded with ^{99m}Tc were injected in mice by tail vain injection. Figure 16 shows blood levels of non-targeted nanoparticles. Figure 17 shows blood levels of targeted nanoparticles. Figure 18 shows bone distribution of non-targeted and targeted nanoparticles. Bars in Figures 16-18 represent mean ± SD (n=6).

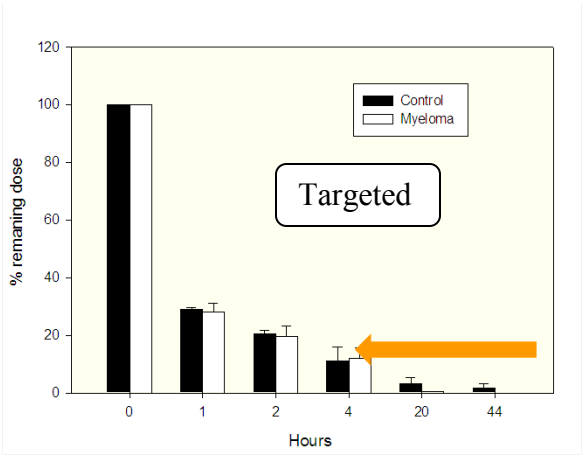

Figure 16. In vivo blood biodistribution in mice (n=6)

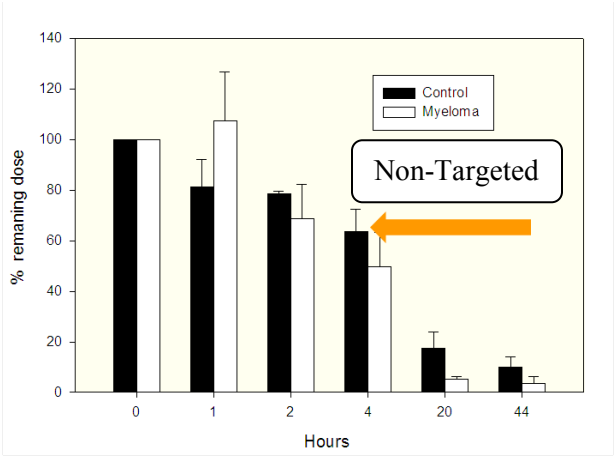

Figure 17. In vivo blood biodistribution in mice (n=6)

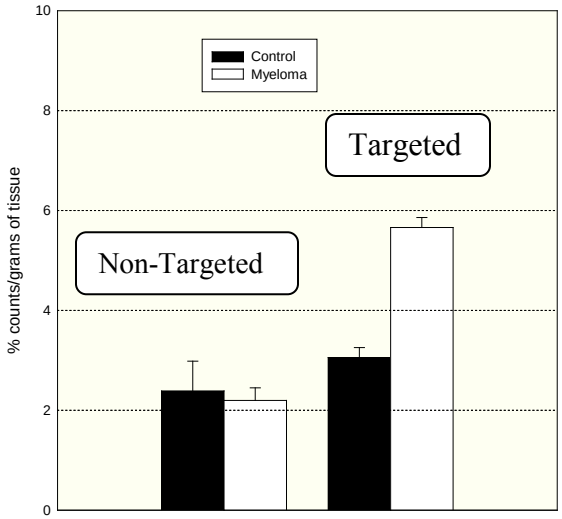

Figure 18. Bone biodistribution in mice (n=6).

3) Task 3 is concerned with demonstrating the efficacy of bone-targeting nanoparticles containing small molecule therapies in a myeloma mouse model. The task is broken down into the following subtasks:

- Prepare radiolabeled bone-targeting nanoparticles, pending
- Conduct *in vivo* efficacy studies, pending.

III. KEY RESEARCH ACCOMPLISHMENTS

- Developed methods to radiolabel polymer nanoparticles; to our knowledge, this is the first time this has been accomplished.
- Quantified functional groups available for ligand conjugation using OPA amino reagent.
- Developed alternative assay to confirm affinity of bone-targeting nanoparticles to hydroxyapatite substrates.
- Demonstrated *in vitro* stability of radiolabeled nanoparticles.
- Successfully transferred our nanoparticle preparation protocols to another facility to support *in vivo* biodistribution studies.
- Started *in vivo* biodistribution studies to validate ability of our radiolabeled nanoparticles to be imaged for up to 48 hours and further confirming our protocol methods to study bone-targeting nanoparticle biodistribution via radio-imaging.

IV. REPORTABLE OUTCOMES

- A change of the principal investigator (PI) was filled this year due to the departure of the previous PI.
- An extension for final completion of this project was granted by the U.S. Army Office. The new completion date is now on March 17, 2010.
- Animal protocols for the biodistribution and efficacy studies described in the original statement of work (Task 2) have been reviewed and approved by the IACUC office and the ACURO army office.
- Manuscript is in preparation describing the development of bone-targeting and radiolabeled nanoparticles.
- Additional professional staff has been hired to support biodistribution and *in vivo* efficacy studies.
- An abstract was submitted and accepted for presentation at the annual CDMRP conference in Kansas City, Missouri in September 2009.

V. CONCLUSIONS

The completed work positions the project to continue biodistribution studies to determine the performance of bone-targeting nanoparticles *in vivo*. We consistently prepare polymer nanoparticles of required size and composition necessary to support other tasks of the project. Technical difficulties encountered during the development of the bone-targeting nanoparticles were due to the principal investigator transfer and the interruptions in the supply of key materials which delayed the project by more than one year. Task 2 remains to be completed, but is expected to be accomplished by late spring and Task 3 would start soon after.

The new SOW will allow us to reduce the number of efficacy studies from 400 to only 100 animals. Under this new efficacy protocol we will look at the efficacy of one proteasome inhibitor formulation instead of multiple drugs.

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APPENDIX I

Abstract was submitted to MFRP Conference. This Military Health Research Forum (MHRF) conference was held at the Hallmark Crown Center in Kansas City, Missouri August 31, 2009 – September 3, 2009. Abstract included below:

BONE-TARGETING NANOPARTICLES FOR TREATMENT OF MYELOMA

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Southwest Research Institute, San Antonio, TX, 78238

BACKGROUND: Multiple myeloma is the second most common adult hematologic malignancy and is unique in its propensity to cause bone destruction [Mundy, '88]. The disease accounts for 1-2% of cancer-related deaths [Jemal, '03] with 80% of patients suffering devastating and progressive bone destruction. Beneficial effects of conventional therapeutic regimens are modest and relapse is invariable, therefore, new treatment strategies are of urgent and vital importance. We have found that several proteasome inhibitors (PI) are effective against both human and murine myeloma cells in culture. Further, we have found the ubiquitin-proteasome pathway exerts exquisite control of osteoblast differentiation and bone formation *in vitro* and *in vivo* in rodents [Garrett, '03]. However, as with any proteasome inhibitor, there are serious concerns over their potential systemic effects and toxicity. Therefore, there is significant need to develop bone-targeted delivery platforms to preferentially deliver these and other drugs to the bone microenvironment.

PURPOSE: The purpose of our work is to determine, in preclinical studies, the potential of skeletally targeted PIs as an efficacious and selective treatment for myeloma. The program hypothesis is that bone-targeting nanocarriers can preferentially accumulate in the skeleton and locally release PIs to impair the capacity of myeloma cells to survive and grow in vivo, thereby reducing the formation and growth of tumor-induced lytic bone lesions. Proteasome inhibitors are not selective to bone and their therapeutic-toxic window may be narrow when administered systemically. Targeted bone delivery has potential to reduce systemic exposure, increase efficacy in the bone environment, and the opportunity to reverse catastrophic disease processes. In this paper we present work on the development and characterization of the bone-targeting nanoparticles that will be used in our preclinical studies.

METHODS: Site-specific targeting requires quantitatively distinct receptors. We selected the calcified matrix as our initial site for bone-targeting. We identified bone-binding ligands and selected two well-known for their predilection to bone surfaces, methylene bisphosphonate (MBP) [e.g. Davis, '76] and an aspartic acid oligopeptide (Asp4) [e.g. Kasugai, '00]. We synthesized amino-MBP and confirmed structure by 1H-NMR. Either aMBP or Asp4 was linked via sulfhydryl-amino conversion to a maleimide-functionalized PEG-b-PLA copolymer. Ligand conjugation was monitored by sulhydryl content using UV-Vis spectroscopy. The PEG-b-PLA copolymer was prepared by ring-opening polymerization of lactide in the presence of a hydroxyl-terminated bifunctional PEG. The block copolymer composition was confirmed by 1H-NMR. Polymer nanoparticles of PLGA/PLA-PEG were prepared by either emulsification/solvent-loss or nanoprecipitation. The particles were purified by ultracentrifugation or cross-flow filtration and lyophilized for long-term storage. Particle size was determined by photon correlation spectroscopy (N4+, Beckman-Coulter) and zeta-potential at 7.0pH (ZetaPALS, Brookhaven). Ligand content was confirmed by amino acid analysis.

RESULTS: We prepared nanoparticles with different compositions ratios of PLGA/PLA-b-PEG using coacervation method and emulsion method with particle size from 100nm to about 200nm, depending on the PEG content. Particle size decreased with increasing PEG content. Similarly, zeta-potential decreased with increasing PEG content, probably due to shielding of the PLGA surface by

the surface PEG groups. Two approaches were used to prepare targeted NPs. The first approach, polymer was synthesized with ligand already on (PLGA-PEG-ASP4) and particles were form after, on the second approach ligand was conjugated after particle formation; the benefit of the first method is reproducibility and speed of processing. Cryoprotectants, such as disaccharides, were required to avoid particle agglomeration during lyophilization. The selected bone-targeting ligands were conjugated to the surfaces of functionalized nanoparticles. Adherence of these ligand-containing nanoparticles to hydroxyapatite substrates was confirmed by radio labeling particles with Tc99m. Cell base assay confirmed the activity of encapsulated drug. Early biodistribution of non targeted NP show prolong blood circulation times with half life around 4 hours

CONCLUSION: We demonstrated the formulation and characterization of nanoparticles for bone-targeting. We showed that these specifically formulated bone-targeting nanoparticles preferentially adhere to bone-like surfaces *in vitro*. *In vivo* biodistribution of two untargeted nanoparticles formulation demonstrate prolong circulation times, encapsulated FDA approved drug Velcade is fully active. This positions us to proceed with in vivo work to study the biodistribution and efficacy of these nanoparticles. We believe this technology has tremendous potential in the treatment of myeloma and other musculoskeletal diseases and disorders.